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GHELIS Charilaos

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devant le Jury :

MM. J. TONNELAT, Président

J. PUDLES

G. COHEN

B. LABOUESSE



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Activity and Conformation of the Alkaline Form of δ -Chymotrypsin Studied by the Specific Acylation of Isoleucine-16*

Charis Ghélis,† Jean-Renaud Garel, and Julie Labouesse‡

ABSTRACT: The N-terminal α -amino group of δ -chymotrypsin was specifically acetylated. During this acetylation, the enzyme showed a loss of activity and a decrease in the concentration of its active sites. The rate of loss of activity was the same as that of disappearance of free isoleucine, while the rate of loss of active sites was much lower. The derivative acetylated on Ile-16 was totally inactive toward specific ester and amide substrates, while it was still capable of hydrolyzing *p*-nitrophenylacetate. It was shown that this hydrolysis took place through a covalent intermediate, presumably an acyl-enzyme. The kinetic analysis of this reaction indicated that the acetylation of Ile-16 led to an appreciable decrease of the acylation rate constant and only to a slight reduction of the deacylation rate constant. Acetylation of

Ile-16 leads to the disruption of the salt bridge Ile-16-Asp-194, in the same way as the deprotonation of Ile-16. The structural properties of these two proteins were very similar as observed by optical rotation, circular dichroism, and titration of exposed tyrosines. The catalytic properties observed for the acetylated enzyme are attributed to the high pH form of chymotrypsin, *i.e.*, a functional active site but no activity toward specific substrates. This absence of activity is probably due to a lack of binding, assigned to a distortion of the binding site. The zymogen was shown to be different from both the acetylated enzyme and the high pH form of chymotrypsin. A two-step activation mechanism is proposed, involving this high pH form as an intermediate between chymotrypsinogen and the active enzyme.

It is now well established that the decrease in the activity of chymotrypsin in the alkaline pH range is linked to the deprotonation of the amino group of its N-terminal residue,

Ile-16 (Oppenheimer *et al.*, 1966; Himoe *et al.*, 1967; Ghélis *et al.*, 1967; Karibian *et al.*, 1968). The X-ray diffraction data describing the three-dimensional structure of the protein have shown that this group is involved in an electrostatic

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‡ To whom correspondence should be addressed.

interaction with the carboxyl group of Asp-194 (Matthews *et al.*, 1967). This interaction keeps the N-terminal amino group inside the molecule, in a hydrophobic environment, and is abolished when the charge is lost. The result of this is a change in the conformation of the protein (Karibian *et al.*, 1968; Mc Conn *et al.*, 1969) and a concomitant decrease in activity toward substrates for which the acylation step is rate limiting (Erlanger and Edel, 1964; Himoe *et al.*, 1967; Karibian *et al.*, 1968; Valenzuela and Bender, 1969). The conformational change, the decrease in activity and the protonic equilibrium of Ile-16 have equal pK 's (Karibian *et al.*, 1968; Garel and Labouesse, 1970), close to 9, and thus should be linked (Tanford, 1961).

It has been proposed that the electrostatic interaction between residues 16 and 194 is critical for the existence of the active structure (Sigler *et al.*, 1968). If this is true, chemical modification of one of these residues, which would prevent it from carrying a charge, should lead to a protein very similar to the high pH form of chymotrypsin. Such a derivative could be studied at neutral pH. By comparing its properties to those of the native enzyme, it should be possible to explain the role of the salt bridge Ile-16-Asp-194 in the active structure.

The specific acetylation of the amino group of Ile-16 is indeed accompanied by a decrease in the activity of the enzyme at optimum pH (Oppenheimer *et al.*, 1966; Ghélis *et al.*, 1967). This decrease in activity may occur for several reasons: (1) the "active site" is destroyed; (2) the "efficiency" of the enzyme is diminished; or, (3) the "affinity" of the enzyme for substrates has decreased.

The present work deals with a detailed study of the effect of the acylation of Ile-16 by acetic, maleic, and citraconic anhydrides on the properties of the enzyme: activity toward specific substrates, reactivity of the active site toward specific and nonspecific titrants, structural features (optical rotation, circular dichroism, and apparent pK 's of exposed tyrosines).

Experimental Section

Materials. Three-times-crystallized chymotrypsinogen, crystallized trypsin and, three-times-crystallized chymotrypsin, came from Worthington. The commercial sources of the following products were: Bz-L-ArgOEt, *N*-Ac-L-TyrOEt, TPCK,¹ from Cyclo; *N*-Ac-L-Trp-NH₂ from Yeda (Israel); DFP, from K & K; NPA, from Merck (Germany); FDNB, from Eastman; *O*-methylisourea sulfate, from Aldrich; Sephadex G-25 and G-50, CM-Sephadex C-50, DEAE-Sephadex A-50, from Pharmacia (Sweden); [³H]DFP (3.6 Ci/mmole), [¹⁴C]acetic anhydride (28 mCi/mmole), from the Radiochemical Centre, Amersham (Great Britain).

Radioactive [*acetyl*-¹⁴C]NPA (14 mCi/mmole) was prepared according to Bender and Nakamura (1962) and NPAT

according to Haugland and Stryer (1967). All other chemicals were of Analytical grade from Prolabo (France) or Merck (Germany).

Methods. PREPARATION OF *N*-ACETYLATED DERIVATIVES. ACTG. Chymotrypsinogen was acetylated in a pH-Stat radiometer (Denmark) type TTT1, and fractionated according to the method of Oppenheimer *et al.* (1966).

ACT. The acetylated zymogen was activated either according to Oppenheimer *et al.* (1966) or at pH 8, 25° by using 1–2 mg/ml of zymogen and 150 μ g/ml of trypsin. In the latter procedure, the activation was stopped when the activity toward *N*-Ac-L-TyrOEt reached a constant value, after about 90 min. In both procedures, the pH of the solution was then lowered to 6. Trypsin was eliminated by batchwise absorption at 0° on CM-Sephadex C-50. The resin was removed by filtration. The filtrate was always tested for tryptic activity to ascertain complete removal of trypsin. The enzyme obtained in this way was fractionated by ammonium sulfate, then dialyzed at 4°. No difference could be found between the final products obtained by either activation procedure.

Estimation of free amino groups showed that the active enzyme was in the δ form of chymotrypsin (Table I). The final yield in ACT was about 80% of the original quantity of ACTG. Both proteins, ACTG and ACT, were stored at –20°, without any loss of potential activity or activity, respectively.

PREPARATION OF *N*-GUANIDINATED DERIVATIVES. Chymotrypsinogen was guanidinated according to Chervenka and Wilcox (1956), at a concentration of protein of 10 mg/ml. After completion of the reaction the solution was dialyzed at 4° against 1 mM HCl to remove the excess of reagent. The extent of guanidination was estimated by reaction of the protein derivatives with FDNB. *N*-Guanidinated δ -chymotrypsin was obtained by activation of *N*-guanidinated chymotrypsinogen in the conditions described for ACT.

SPECIFIC ACYLATION OF THE AMINO GROUP OF *N*-TERMINAL ISOLEUCINE. The specific acetylation of the amino group of ACT was performed with radioactive [¹⁴C]acetic anhydride (28 mCi/mmole), at a concentration of protein between 2 and 4 mg per ml under conditions described previously (Ghélis *et al.*, 1967). The reagent was added over a period of 40 min. The reaction mixture was kept at the same pH until complete hydrolysis of the anhydride.

Aliquots of the reaction mixture were taken at given time intervals and the incorporation of [¹⁴C]acetyl into the protein, the activity, and the concentration of enzyme active sites were followed. The samples were treated with 0.5 M hydroxylamine at pH 7.5 and 25° for 30 min to stop the reaction and to deacetylate eventually blocked hydroxyl and imidazolyl groups of the protein. The samples were then exhaustively dialyzed 4 times with 5 l. of 10 mM phosphate buffer at pH 7 (1 hr at 25°, then 36–48 hr at 4°). The protein solutions which were turbid at the end of the dialysis were centrifuged for 1 hr at 0° and 35,000g. All properties of a given sample of A₁₆ACT were examined within 3 days (radioactivity, enzymic activity, concentration of active sites, free amino groups, optical rotation, and circular dichroism).

The reaction of Ile-16 with maleic anhydride was performed according to Butler *et al.* (1969) at a final concentration of 1% of reagent, 2 mg/ml of protein, in 4% (v/v) dioxane.

¹ Abbreviations used are: TPCK, L-1-tosylamino-2-phenyl ethyl chloromethyl ketone; NPA, *p*-nitrophenylacetate; NPAT, *p*-nitrophenylanthranilate; ACTG, chymotrypsinogen in which all of the ϵ -amino groups of lysine residues and the α -amino group of half-cystine residue are acetylated; ACT, δ -chymotrypsin obtained by activation of *N*-acetylchymotrypsinogen, ACTG, in which the only free α -amino group is that of the new N-terminal Ile-16; A₁₆ACT, derivative obtained from *N*-acetyl- δ -chymotrypsin, ACT, by specific acetylation of the α -amino group of N-terminal isoleucine.



TABLE I: Determination of Free Amino Groups in ACTG, ACT, and during Acetylation of ACT and Acetyl- α -chymotrypsin.

	Acetylation Time (min)	Free α -NH ₂ ^c Ile (res/mole)	Free α -NH ₂ ^c Ala (res/mole)	Nonidentified Free NH ₂ (res/mole)	Incorporated ^d [¹⁴ C]Acetyl (res/mole)
ACTG		0	0		
ACT ^a		0.95	0.1	0.1	
ACT ^b	0	0.95	<0.1	0.1-0.2	0
	15	0.45	<0.1		0.8
	40	0.15			1.0
	60	0.10	<0.1	<0.1	1.1
Acetyl- α -chymotrypsin ^e	0	0.95	1.1		0
	40	0.10	0.8		1.25

^a Before treatment with hydroxylamine. ^b After treatment with 0.5 M hydroxylamine, 30 min, 25°, pH 7.5. ^c The results are given with an accuracy of ± 0.1 residue (res) per mole. Thus values between 0 and 0.1 are given as <0.1, except for ACTG for which no free amino group could be detected. ^d ± 0.05 [¹⁴C]acetyl/mole. ^e Obtained by activation of ACTG.

The reaction of Ile-16 with citraconic anhydride was performed according to Dixon and Perham (1968), at a final concentration of 1% of reagent, and 2 mg/ml of protein. The citraconyl-amino bond is rather labile (Dixon and Perham, 1968). The solutions of enzyme derivatives were therefore always stocked at pH 6.5 to 7.

The proteins were not treated by hydroxylamine after reaction with dicarboxylic anhydrides.

PROTEIN CONCENTRATION. The protein concentrations were determined spectrophotometrically on a Cary 15 spectrophotometer, at 280 nm, using the following molar extinction coefficients: $\epsilon_{M_{280}}$ 5×10^4 M⁻¹ cm⁻¹ for chymotrypsinogen, α -chymotrypsin, A₁₆ACT treated with hydroxylamine, N-guanidinated chymotrypsinogen, and δ -chymotrypsin; $\epsilon_{M_{280}}$ 4.7×10^4 M⁻¹ cm⁻¹ for the acetylated derivatives not treated with hydroxylamine (Oppenheimer *et al.*, 1966).

ENZYMIC ACTIVITY. The activity observed for a sample was always referred to its concentration of protein as determined by its absorbancy at 280 nm. The rate constants were expressed in sec⁻¹.

The esterase activity of enzyme derivatives was determined toward N-Ac-L-TyrOEt, according to Schwert and Takenaka (1955), under the following conditions: 2-15 μ g/ml of enzyme depending upon the activity of the sample, in 150 mM KCl, pH 8 at 25°. The amidasic activity was determined toward N-Ac-L-Trp-NH₂, by measuring the rate of release of ammonia according to Moore and Stein (1948), under the following conditions: 50 mM pyrophosphate buffer, 50-250 μ g/ml of enzyme depending upon its activity, in 150 mM KCl, pH 8 at 25°.

The test of tryptic activity in activation mixtures was carried out in a pH-Stat, with 5 mM Bz-L-ArgOEt, in 100 mM NaCl, at pH 8 and 25°.

CONCENTRATION OF ACTIVE SITES. The concentration of active sites of enzyme derivatives was determined by several methods.

The routine determination was done by measuring the "burst" of *p*-nitrophenol upon hydrolysis of NPA in a Cary

15 recording spectrophotometer. Two sets of conditions were used: (a) pH 8.5, 25°, 100 mM pyrophosphate buffer, at 400 nm where the molar extinction coefficient of *p*-nitrophenolate ion is ϵ_M 1.8×10^4 M⁻¹ cm⁻¹ (Bender *et al.*, 1966a); (b) pH 6.5, 15°, 100 mM phosphate buffer, at the isosbestic point of *p*-nitrophenol and *p*-nitrophenolate ion, 347.5 nm, using a molar extinction coefficient ϵ_M 5.3×10^3 M⁻¹ cm⁻¹ (Lawson and Schramm, 1965). Both conditions were used with concentrations of enzyme ranging from 5 to 20 μ M, depending upon the size of the burst and a concentration of NPA of 1 mM (3% (v/v) acetonitrile). Both methods gave very similar results. The rise of absorbance was recorded until the steady state release of *p*-nitrophenol was reached, at least for 10 min.

Titration of active sites was also performed by reaction with [³H]DFP (3.60 mCi/mmol) at pH 7, 25°, 10 mM DFP, 40-80 μ M enzyme in 100 mM phosphate buffer, 0.6% (v/v) 2-propanol. When the kinetics of this reaction was studied, aliquots were taken and immediately "sieved" through Sephadex G-25 (fine) to stop the reaction (20 $\times$ 2 cm columns equilibrated with 20 mM phosphate buffer, pH 7). The protein fractions were exhaustively dialyzed against the same buffer.

The titration by hydrolysis of NPAT was performed spectrophotometrically at 400 nm according to Haugland and Stryer (1967).

The titration by hydrolysis of NPA was also performed with [acetyl-¹⁴C]NPA, to show the formation of a covalent intermediate. At the end of the spectrophotometric titration, the protein was precipitated by addition of trichloroacetic acid up to a final concentration of 5%. The precipitate was centrifuged at 0°, washed twice with 5% trichloroacetic acid, then twice with ethanol to remove traces of unreacted [¹⁴C]NPA, free acetic acid, or *p*-nitrophenol. The protein was then dissolved in 100 mM NaOH and radioactivity was measured in a liquid scintillation spectrometer. The amount of protein was estimated spectrophotometrically using a solution of ACT of known concentration in 100 mM NaOH as reference.

The concentrations of active sites obtained for various

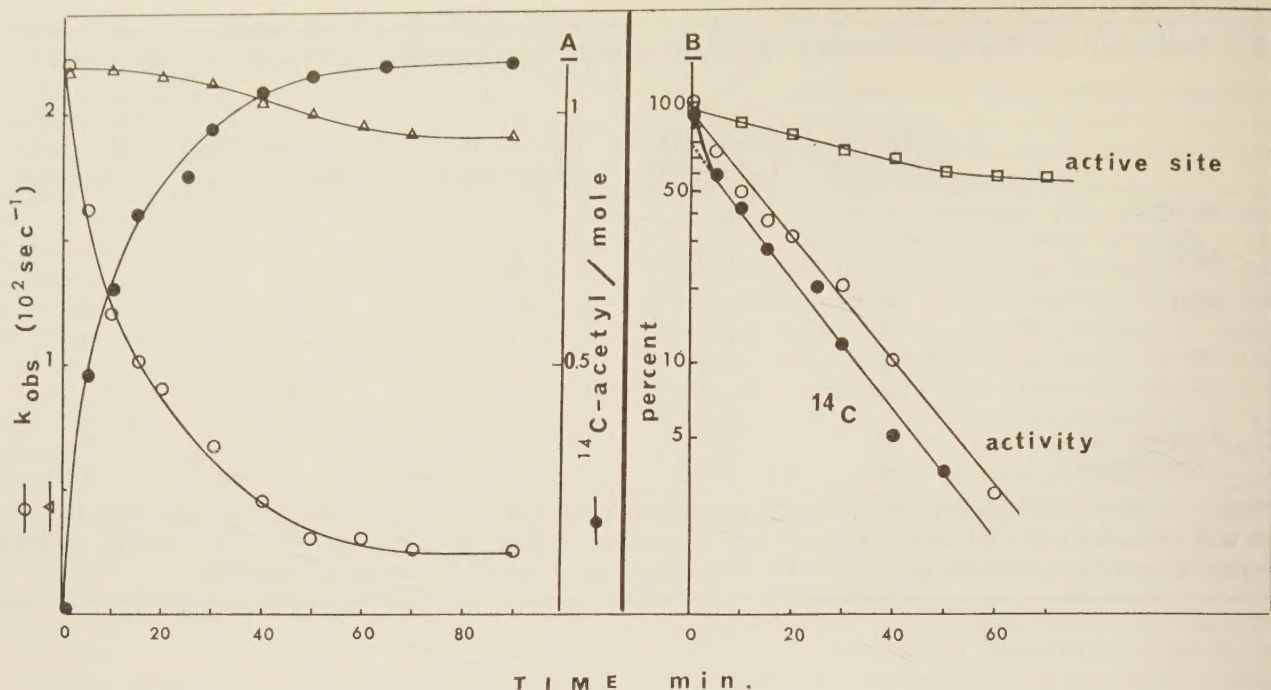


FIGURE 1: Acetylation of Ile-16 of ACT. (A) Time dependence of ^{14}C incorporation (●); activity toward *N*-Ac-L-TyrOEt during acetylation (○) and blank reaction (Δ). (B) First-order plot of loss of activity toward *N*-Ac-L-TyrOEt (○), ^{14}C incorporation (●), and of loss of active sites (values of curve c, Figure 2) (□).

derivatives were calculated from the spectrophotometrically determined protein concentration, unless otherwise specified.

DETERMINATION OF SPECIFIC RADIOACTIVITIES. The radioactivities of ^{14}C and ^3H were estimated by the method of Bray (1960), and corrected for quenching by the protein in a liquid scintillation counter (Nuclear-Chicago Mark I).

ESTIMATION OF FREE AMINO GROUPS. Reaction with FDNB (Phillips, 1958) was used to determine the nature and quantity of free α - and ϵ -amino groups in enzyme derivatives. The quantitative determination was performed according to Labouesse and Gervais (1967) on samples of protein of the order of 10 mg. The following solvents were used for thin-layer chromatography of the ether-soluble DNP-amino acids: 1st dimension, chloroform-benzyl alcohol-glacial acetic acid (70:30:3); 2nd dimension, toluene-pyridine-monochloroethanol-25% ammonia (100:15:30:5). The number of free amino groups was evaluated to ± 0.1 residue per mole of protein.

OPTICAL ROTATION AND CIRCULAR DICHROISM. Optical rotation of enzyme solutions was measured at 313 nm in a Perkin-Elmer polarimeter 141, equipped with a jacketed cell of 10-cm optical path length, thermostated at 25°. Ellipticity of enzyme solutions was measured in a Roussel-Jouan dichrograph II at room temperature. The protein solutions were centrifuged at 0°, 35,000*g*, for 1 hr before measurements. The stock enzyme solutions were diluted just before use in phosphate or carbonate buffer, to the desired pH. The final concentrations were: 20 mM buffer-150 mM KCl-1 mg/ml of protein for polarimetric and 0.5 mg/ml for dichroic measurements. The final pH was determined immediately after each measurement.

CHROMATOGRAPHY OF A₁₆ACT. Purification of A₁₆ACT was attempted either by sieving through Sephadex G-25

at pH 6.5 in water or by chromatography on DEAE-Sephadex C-50 at pH 7.5 in 20 mM Tris-HCl buffer, followed by stepwise elution with KCl up to 0.5 M.

REACTIVATION OF CHYMOTRYPSIN DERIVATIVES SPECIFICALLY BLOCKED IN THE α -AMINO GROUP OF ISOLEUCINE. The reactivation of A₁₆ACT was achieved by treating the protein with 4 M hydroxylamine at pH 7.5, at 25°. All the properties of the reactivated enzyme were determined on aliquots of the reactivation mixture, which were first diluted 6 times and then dialyzed exhaustively at 4° against 10 mM phosphate buffer at pH 7.

The enzyme, the N-terminal α -amino group of which had been blocked by reaction with citraconic anhydride, was reactivated at pH 3, at 25°, immediately after the acylation reaction. The activity of the samples toward *N*-Ac-L-TyrOEt was determined without prior dialysis.

Results

*Time Course of Specific Acetylation of the N-Terminal Isoleucine in ACT. Incorporation of [^{14}C]Acetyl, Loss of Activity toward *N*-Ac-L-TyrOEt and of Free α -Amino Groups.* It has been shown previously (Ghéris *et al.*, 1967) that the conditions of acetylation of ACT used in this work lead to the specific labeling of the α -amino group of N-terminal isoleucine.

Figure 1A shows the kinetics of incorporation of [^{14}C]acetyl into ACT and that of loss of activity toward *N*-Ac-L-TyrOEt. The same figure gives also the evolution of the activity of an acetylation blank in which glacial acetic acid, instead of acetic anhydride, was added to ACT at the same rate and up to the same final concentration of acetate. This treatment brings about some inactivation, probably due to

TABLE II: Kinetic Parameters of ACT and A₁₆ACT for Specific Substrates.

	<i>N</i> -Ac-L-TyrOEt ^a		<i>N</i> -Ac-L-Trp-NH ₂ ^b	
	$k_{\text{obsd,max}}$ (sec ⁻¹)	$K_{\text{m,app}}$ (mM)	$k_{\text{obsd,max}} \times 10^3$ (sec ⁻¹)	$K_{\text{m,app}}$ (mM)
ACT	235 ± 5	0.8 ± 0.2	36 ± 2	4 ± 1
A ₁₆ ACT	22 ± 5	1.3 ± 0.2	3 ± 2	4 ± 1

^a pH 8, 150 mM KCl, 25°; *N*-Ac-L-TyrOEt, 0.2–30 mM. ^b 50 mM pyrophosphate buffer, pH 8, 150 mM KCl, 25°; *N*-Ac-L-Trp-NH₂, 2–8 mM.

denaturation. Therefore during acetylation the observed activities were corrected for the nonspecific inactivation estimated from the blank reaction. The corrected values were used to compare the rate of loss of activity with that of ¹⁴C incorporation (Figure 1B). The ¹⁴C incorporation appeared to be a two-step reaction (Figure 1B). The first step led to the rapid labeling of about 0.2 residue per mole of protein, corresponding probably to the extra free amino groups found in ACT (Table I). The second slow step corresponded to the incorporation of 0.9 residue of [¹⁴C]acetyl per mole of protein and its rate was very comparable to that of the loss of activity. This figure of 0.9 was in accordance with the amount of free isoleucine disappearing upon acetylation, 0.9 residue/mole, as titrated by FDNB (Table I)

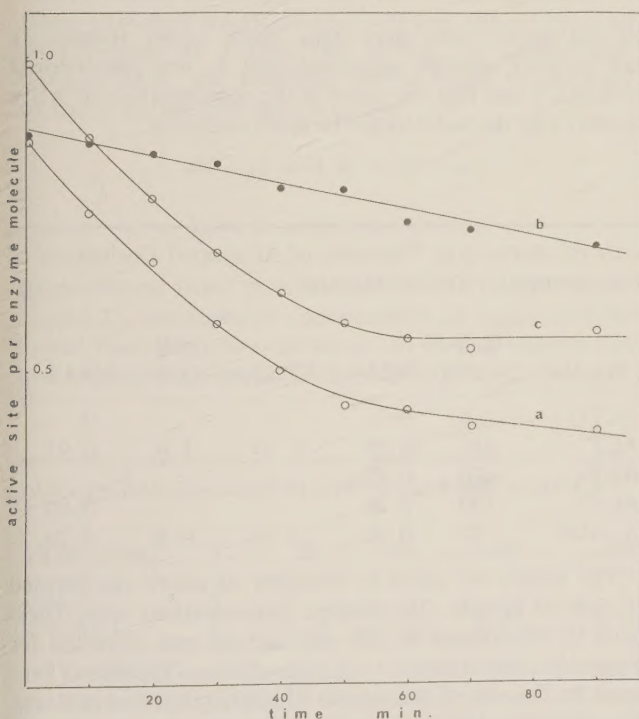


FIGURE 2: Time dependence of loss of active sites during acetylation of Ile-16 of ACT: (a) acetylation reaction; (b) blank reaction; (c) loss of active site during acetylation corrected for unspecific denaturation. (Ratio of values of curve a to values of curve b). The concentrations of active sites were determined by titration with NPA in either set of standard conditions.

Thus, the loss of activity toward *N*-Ac-L-TyrOEt could be correlated to the specific acetylation of Ile-16.

Original ACT contained 0.1 residue of free N-terminal alanine. This content remained constant during acetylation and could not be involved in the final balance of free amino groups and [¹⁴C]acetyl incorporation, since the amino group of N-terminal alanine of α -chymotrypsin reacts slowly with acetic anhydride (Table I).

The final product, A₁₆ACT, had 11% of the original activity and 10% of free N-terminal isoleucine.

Loss of Active Sites during Acetylation of Ile-16. The kinetics of the decrease of concentration of ACT active sites was followed for the acetylation reaction and for its blank (Figure 2). In the blank reaction the loss of ACT active sites was comparable to the loss of activity toward *N*-Ac-L-TyrOEt. The rate of loss of active sites during acetylation of Ile-16 was determined after correction for nonspecific denaturation (Figure 1B). This rate was significantly lower than that of loss of activity and of incorporation of ¹⁴C into the protein, leading to a final product, A₁₆ACT, with more than 50% of active site. It has to be mentioned here that all measurements were performed on samples treated with hydroxylamine (see Experimental Section), in order to remove all labile acetyl groups.

A₁₆ACT showed more than 50% of active site as opposed to about 10% of activity toward *N*-Ac-L-TyrOEt; this suggested that A₁₆ACT behaved differently toward specific and nonspecific substrates.

Activity of A₁₆ACT toward Specific Substrates. The residual activity of A₁₆ACT toward two specific substrates was studied in the following ranges of concentrations: 0.2–30 mM for *N*-Ac-TyrOEt and 2–8 mM for *N*-Ac-L-Trp-NH₂ (the upper concentrations correspond to the limit of solubility in water of both substrates). The maximum rate, $k_{\text{obsd,max}}$, of hydrolysis of both substrates by A₁₆ACT was about 10–12% of that by original ACT. The apparent Michaelis constants, $K_{\text{m,app}}$, were comparable for A₁₆ACT and ACT (Table II). This supported the conclusion that the observed activity of A₁₆ACT was due to the nonacetylated fraction of ACT (10% of original sample, Table I).

This amount of residual ACT could be lessened by longer acetylation times, but with concomitant larger denaturation. For this reason the study of A₁₆ACT acetylated for 40 min was preferred, though this product was a mixture of 10% of nonacetylated active ACT and 90% of protein acetylated on Ile-16. This latter fraction was considered as inactive toward specific substrates.

Reactivity and Kinetic Properties of the Active Site of A_{16} ACT. The acetylated fraction of A_{16} ACT was found to be partially active toward NPA, since more than 50% of active site of A_{16} ACT could not be assigned to 10% of residual ACT. Several reasons could explain this partial activity. (1) Denaturation of the protein might be higher than that estimated from the reaction blank: as a matter of fact A_{16} ACT was less stable than ACT; aged preparations lost progressively their capacity to hydrolyze NPA. All purification efforts to eliminate the fraction inactive toward NPA were unsuccessful. (2) Nonspecific catalysis of hydrolysis of NPA by the protein: to check that this was not the case, the titration was performed with radioactive [acetyl- 14 C]NPA. A labeled protein could be isolated (Table III), which indicated that the hydrolytic reaction went through a covalent intermediate. This intermediate could have been labeled on a histidine residue, but the acetyl-imidazolyl bond would not have withstood the isolation in 5% trichloroacetic acid (Bruce and Benkovic, 1966). The isolated intermediate was likely acetyl-chymotrypsin, acylated on Ser-195.

It was also checked that under the same conditions ACTG did not catalyze appreciably the hydrolysis of NPA. Moreover, the burst of *p*-nitrophenol was proportional to the concentration of A_{16} ACT (Figure 3). Thus it was assumed that A_{16} ACT behaved like the normal enzyme toward NPA. (3) Lack of saturation of A_{16} ACT by NPA: a change of concentration of NPA in the range of 0.5–5 mM affected neither the shape nor the size of the "burst." Under standard titration conditions, A_{16} ACT was saturated by NPA. The classical scheme of the acyl-enzyme was considered to be applicable to A_{16} ACT. (4) Change in the ratio of the kinetic constants of acylation, k_2 , and deacylation, k_3 : the relationship between the burst and the actual concentration of active sites was derived from the acyl-enzyme scheme by Bender *et al.* (1966a). At substrate saturation the burst, π , corresponding to a concentration of active site, E_0 , is given by

$$\pi = E_0 \left[\frac{k_2}{k_2 + k_3} \right]^2$$

The rate constants k of the exponential phase and k_{ss} of the steady state are

$$k = k_2 + k_3$$

$$k_{ss} = E_0 \frac{k_2 k_3}{k_2 + k_3}$$

The burst is equal to the actual concentration of active sites only when $k_2 \gg k_3$. For instance the value determined for ACT at pH 8 and 25° were $k_2 = 3.5 \text{ sec}^{-1}$ (C. Ghélis, 1969, unpublished results), and $k_3 = 3 \times 10^{-2} \text{ sec}^{-1}$ (Figure 4), in fairly good agreement with values found for α -chymotrypsin (Gutfreund and Sturtevant, 1956; Fallor and Sturtevant, 1966; Bender *et al.*, 1966b). If the condition $k_2 \gg k_3$ is not fulfilled, the observed burst π leads to an apparent value of the concentration of active sites which is lower than is actually the case, and k_{ss} is then also lower than k_3 (Bender *et al.*, 1966a).

The release of *p*-nitrophenol during hydrolysis of NPA by A_{16} ACT and ACT was compared; the exponential phase

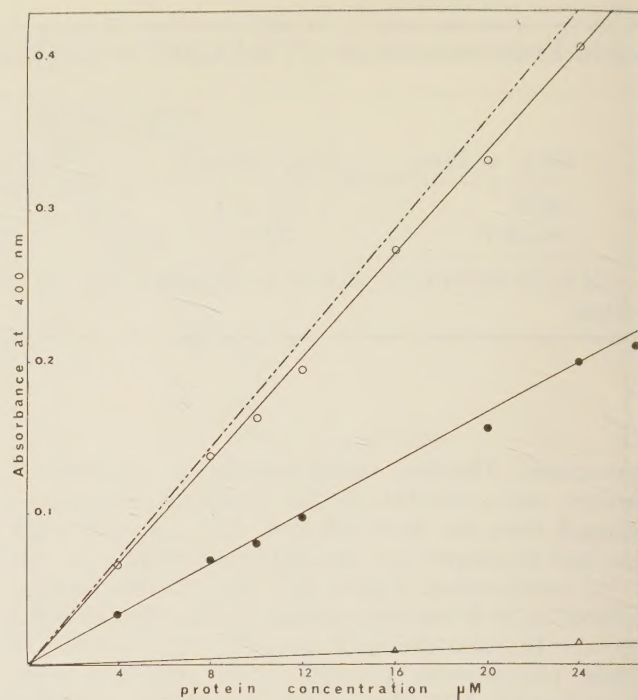


FIGURE 3: Effect of protein concentration on the amplitude of the observed burst in titrations of active sites with NPA: (O) ACT not treated with hydroxylamine; (●) A_{16} ACT; (Δ) ACTG treated with hydroxylamine in the same conditions as A_{16} ACT. Titrations in standard conditions as described in text. The dashed line corresponds to 100% of active site per mole of protein.

and the steady state were both much slower (Figure 4). Such kinetics strongly suggested that k_2 was much lower for A_{16} ACT and that the value of the concentration of active sites given by the burst might be underestimated.

TABLE III: Active Site Titrations of Acetylated Derivatives of Chymotrypsin by Various Methods.^a

Protein	k_{obsd}^b (sec ⁻¹)	NPA ^c	[14 C]NPA ^d	[3 H]- DFP ^e	NPAT ^{e,f}
ACTG	0	0			0
ACT	235	0.95	0.70	1.0	0.95
ACT ^g	220	0.85			
ACT ^h	190	0.70			0.80
A_{16} ACT	22	0.40	0.36	0.8 ^f	0.14

^a The results are given in fractions of active site titrated per mole of protein. The enzyme concentrations were determined by absorbancy at 280 nm, and are not corrected for nonspecific inactivation. ^b k_{obsd} for *N*-Ac-L-TyrOEt. ^c Estimated by the size of the release of *p*-nitrophenol at 400 nm. ^d Estimated by 14 C incorporation into the protein after 10 mn of reaction. ^e Estimated by 3 H incorporation into the protein. ^f End of blank reaction (90 mn). For experimental details, see text. ^g Treated with 0.5 M hydroxylamine, 30 mn, 25°, pH 7.5, and dialyzed. ^h After 12 hr of reaction (Table IV).

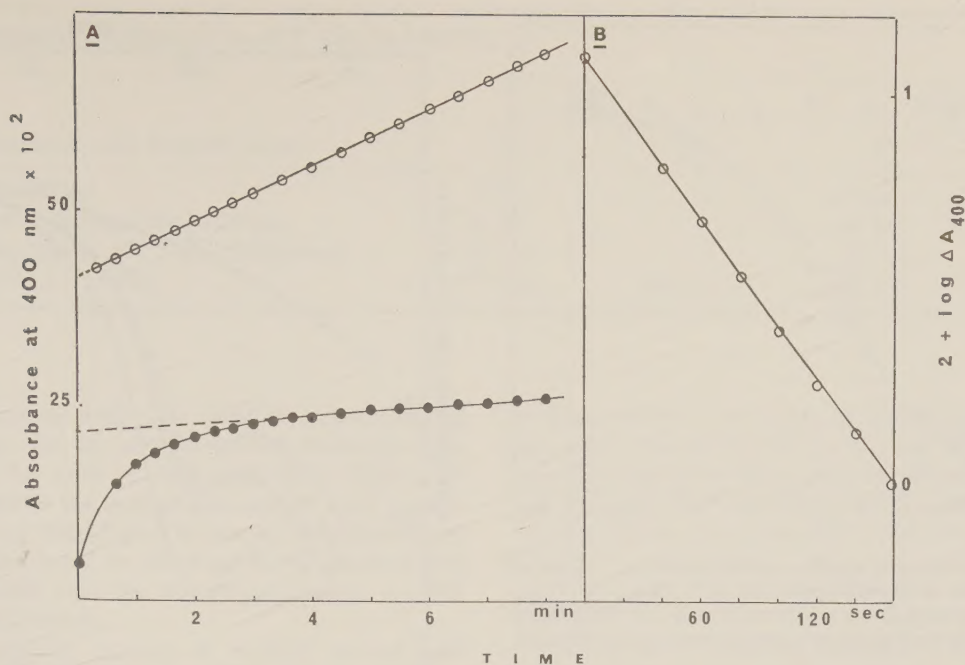


FIGURE 4: Kinetics of release of *p*-nitrophenolate ion during titration of active sites with NPA, after correction for spontaneous hydrolysis: (A) (○) ACT, (●) A₁₆ACT; (B) first-order plot of the presteady-state release of *p*-nitrophenolate ion in the case of A₁₆ACT. The extrapolation of this plot at zero time was used to calculate the ordinate intercept in Figure 4A. The amount of residual ACT (about 10%) was determined from the amplitude of the instantaneous burst. Conditions of titration: 1 mM NPA, 24 μ M enzyme, pyrophosphate buffer 100 mM, pH 8.5, 25°, 3% (v/v) acetonitrile.

A tentative analysis of the burst observed for A₁₆ACT was undertaken, assuming that A₁₆ACT contained two fractions, a fraction acetylated on Ile-16 and about 10% residual ACT (Figure 4). Taking into account the contribution of residual ACT, the following values could be assigned to k_2 and k_3 for the acetylated fraction

$$k_2 = (2 \pm 0.4) \times 10^{-2} \text{ sec}^{-1}$$

$$k_3 = (1 \pm 0.4) \times 10^{-2} \text{ sec}^{-1}$$

(mean values from 11 experiments). With such values for k_2 and k_3 , the apparent concentration of active sites determined from the observed burst was in fact underestimated and should correspond to approximately one-half of the

actual concentration. At least 80% of the fraction acetylated on Ile-16 should be active toward NPA.

To corroborate this interpretation the active site of A₁₆ACT was titrated with [³H]DFP by measuring the incorporation of ³H into the protein. This reaction was also much slower for A₁₆ACT than for ACT and led to the incorporation of 0.8 [³H]diisopropylphosphoryl residue/mole of A₁₆ACT (Table IV). The amount of inert protein in A₁₆ACT was of the order of 20% in both titrations, in agreement with the extent of denaturation estimated from the reaction blank.

However, only the residual ACT could be titrated in A₁₆ACT by NPAT, a bulkier and more specific substrate (Haugland and Stryer, 1967) (Table III). Similarly the protein acetylated on Ile-16 did not react with TPCK under the conditions described by Schoellmann and Shaw (1962).

Though active toward NPA and DFP, chymotrypsin acetylated on Ile-16 was indeed inactive toward specific substrates and pseudosubstrates.

Structural Studies of A₁₆ACT. OPTICAL ROTATION. The alkaline transition of chymotrypsin linked to the ionization of the α -amino group of Ile-16 (Oppenheimer *et al.*, 1966; Karibian *et al.*, 1968; Mc Conn *et al.*, 1969; Garel and Labouesse, 1970) was no longer observed when this group was acetylated (Figure 5). A₁₆ACT had a specific rotation very close to that of the alkaline form of ACT. It could be noted that the apparent p*K* observed for the transition of ACT was 8.3 at 25° (Figure 5) and 9 at 15° (Karibian *et al.*, 1968; Garel and Labouesse, 1970). Such a large ΔpK indicated that the α -amino group of Ile-16 involved in this transition was far from normal (Edsall and Wyman, 1958).

CIRCULAR DICHROISM. The circular dichroic spectra between 220 and 250 nm of ACTG, ACT, and A₁₆ACT were compared

TABLE IV: Reaction with [³H]FDP of ACT and A₁₆ACT.^a

Time (min)	0	20	60	120	720
ACT	0	1			1.1
A ₁₆ ACT	0	0.28	0.32	0.43	0.80

^a The results, not corrected for nonspecific inactivation, are given in residue of [³H]DIP incorporated per mole of protein with an accuracy of ± 0.05 residue/mole. Protein concentration were determined by absorbancy at 280 nm: 10 mM DFP, 0.6% (v/v) 2-propanol, 40–80 μ M enzyme, 100 mM phosphate buffer, pH 7, 25°. Excess of DFP was eliminated by gel filtration and dialysis (see text).

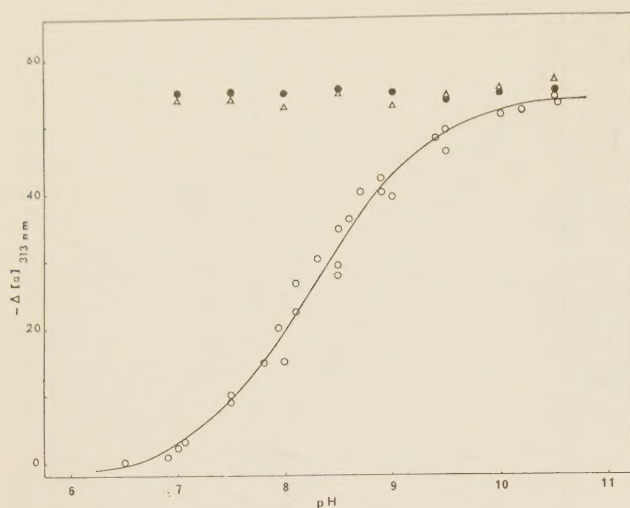


FIGURE 5: pH dependence of specific optical rotation at 313 nm of acetylated derivatives of chymotrypsin: (O) ACT; (●) A₁₆ACT; (Δ) ACTG. Protein concentration ranged from 39 to 46 μ M; temperature 25°. The solid line is the theoretical curve for one group with a pK equal to 8.3.

(Figure 6). ACT showed a minimum centered at 229–230 nm, the intensity of which changed between pH 7 and pH 10, in agreement with results obtained for α -chymotrypsin (Mc Conn *et al.*, 1969; Volini and Tobias, 1969). The negative dichroic band of ACT at pH 7 at 230 nm was slightly smaller than that of α -chymotrypsin (Mc Conn *et al.*, 1969; E. Schechter, 1969, personal communication) and that of δ -chymotrypsin (J. Labouesse, 1969, unpublished results) under identical conditions. The spectrum of A₁₆ACT was not sensitive to pH. Though A₁₆ACT was shown to be a mixture, the shape of its dichroic spectrum suggested that the structure of the fraction acetylated on Ile-16 was similar to the high pH form of ACT and to ACTG. In fact, the presence of some residual ACT and of some denatured material should have opposite contributions on the observed ellipticity. The structural analogy between A₁₆ACT, ACTG, and the high pH form of ACT presented here is in qualitative agreement with the results of Hess *et al.* (1970).

TITRATION OF EXPOSED TYROSINES. The two exposed tyrosines of ACTG and ACT have been titrated previously (Karibian *et al.*, 1968). The pK's of exposed tyrosines determined for ACT necessarily imply the high pH form. Both exposed tyrosines have different pK's in ACTG and in ACT, respectively. The same residues were titrated in A₁₆ACT according to Karibian *et al.* (1968) (Figure 7) and were found to be significantly different from those of ACTG. In no case could this difference be explained by the presence of only 20% of denatured material. But the presence of such material could perhaps account for the shift of the titration curve of A₁₆ACT from that of ACT.

Deacetylation and Reactivation of A₁₆ACT. The nucleophilic attack by hydroxylamine of amide bonds in conditions in which ester bonds are cleaved (0.5–1 M NH₂OH) was not appreciable. Very few N-terminal amino acids appeared in an N-acetylated protein upon treatment with 0.5 M NH₂OH (Table I). Nevertheless, amide bonds can be cleaved by

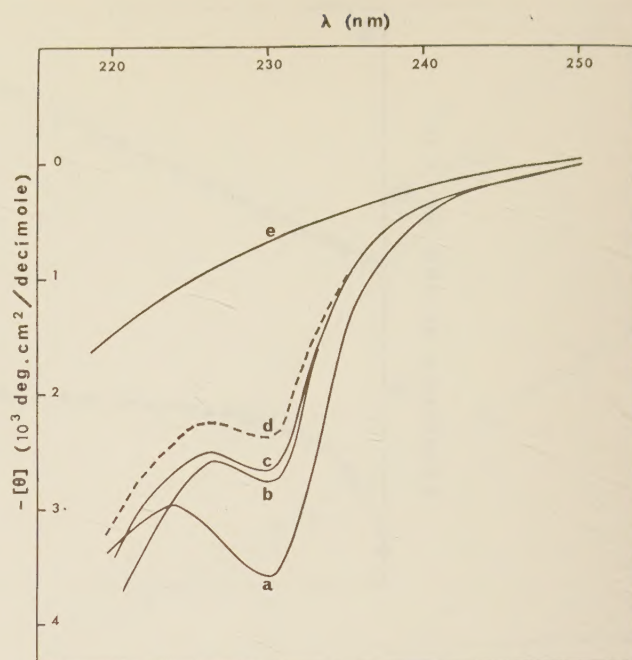


FIGURE 6: Circular dichroic spectra of acetylated derivatives of chymotrypsin: (a) ACT, pH 7.2; (b) ACTG, pH 7.2 and 9.9; (c) ACT, pH 9.9; (d) A₁₆ACT, pH 7.2 and 9.9; (e) ACT and A₁₆ACT in 8 M urea at pH 7.2

hydroxylamine under more drastic conditions (Katz *et al.*, 1953; Jencks and Gilchrist, 1964).

Treatment with 4 M NH₂OH at pH 7 and 25° of A₁₆ACT (labeled with [¹⁴C]acetyl on its Ile-16) led to the loss of ¹⁴C from the protein (Figure 8) and to an increase in free N-terminal isoleucine (Table V). After 25 min of this treatment the enzyme recovered nearly 50% of the activity of original ACT toward N-Ac-L-TyrOEt (Figure 8). Kinetics of release of *p*-nitrophenol upon hydrolysis of NPA by this reactivated enzyme was comparable to that observed for ACT. The burst corresponded to 50% of functional active site.

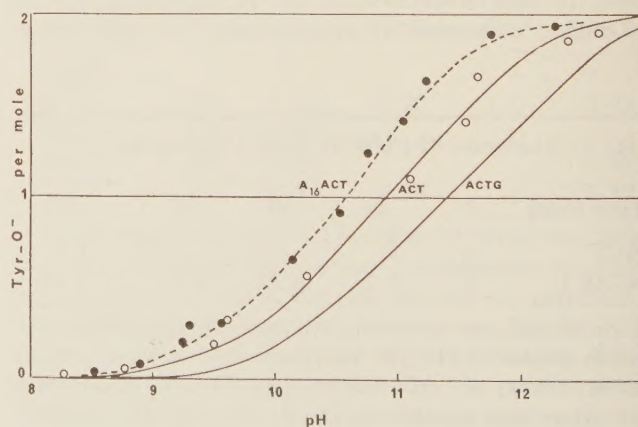


FIGURE 7: Spectrophotometric titration of the two exposed tyrosine residues in ACT and A₁₆ACT: (O) ACT; (●) A₁₆ACT. Solid curves correspond to the titration curves obtained by Karibian *et al.* (1968) for ACT and ACTG.

TABLE V: Reactivation by Treatment of A₁₆ACT with 4 M NH₂OH.

Time of Reaction with NH ₂ OH (min)	ACT		A ₁₆ ACT	
	0	25	0	25
Active site/mole (NPA)	0.9		0.40	0.40
Free N-terminal isoleucine (residue/mole)	0.95		0.10	<0.5
Optical rotation, $\Delta[\alpha]_{313}$, pH 7–pH 10 (degrees)	54 ± 3		2 ± 3	30 ± 3
k_{obsd} (sec ⁻¹) (<i>N</i> -Ac-L-TyrOEt)	235 ± 5	205 ± 5	23 ± 5	120 ± 5

When the treatment with 4 M NH₂OH was performed for more than 30 min the activity toward *N*-Ac-L-TyrOEt again decreased for both A₁₆ACT and ACT (Figure 8). This might be due to the gradual cleavage of some peptide bonds under these conditions. It has to be remembered that no reactivation could be observed, during the treatment with 0.5 M NH₂OH used for the preparation of A₁₆ACT (see Experimental Section).

The specific optical rotation of A₁₆ACT treated with 4 M NH₂OH during 25 min was again sensitive to pH, in the pH range 7–10 (Table V). This could be related to the fact that Ile-16 was again free to ionize; but some amino groups eventually liberated by hydroxylaminolysis might contribute to these changes in optical rotation.

Acylation of Ile-16 by Other Substituents. The chemical nature of the group substituting either the ϵ -amino groups of lysine residues or the α -amino group of Ile-16 had no effect on the modification of properties of chymotrypsin linked to the acylation of its N-terminal residue. The properties of N-guanidinated δ -chymotrypsin were very similar to those of ACT. N-Guanidinated δ -chymotrypsin was reacted with either maleic or citraconic anhydride. Both derivatives

acylated on Ile-16 showed a behavior comparable with that of A₁₆ACT toward *N*-Ac-L-TyrOEt and NPA. The reactivation of the citraconyl derivative was achieved at pH 3 (Dixon and Perham, 1968) and led to the recovery of 75% of the original activity toward *N*-Ac-L-TyrOEt (Figure 9).

Discussion

The aim of this work was to prepare a chemical derivative of chymotrypsin, in which the α -amino group of Ile-16 would be specifically acylated, in order to determine the properties of the enzyme in the "alkaline" conformation. The main results were obtained with an enzyme acetylated on its Ile-16. It was checked that the chemical nature of the acylating group of Ile-16 had no effect on the properties of the modified protein, by using N-guanidinated chymotrypsin and maleic or citraconic anhydrides.

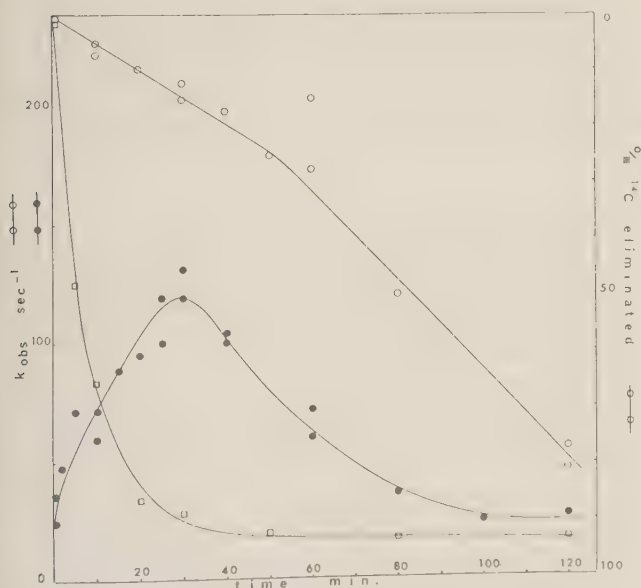


FIGURE 8: Reactivation of A₁₆ACT during treatment with 4 M hydroxylamine: (●) activity of A₁₆ACT (*N*-Ac-L-TyrOEt); (○) activity of ACT (*N*-Ac-L-TyrOEt); (□) loss of [¹⁴C]acetyl from [¹⁴C]A₁₆ACT.

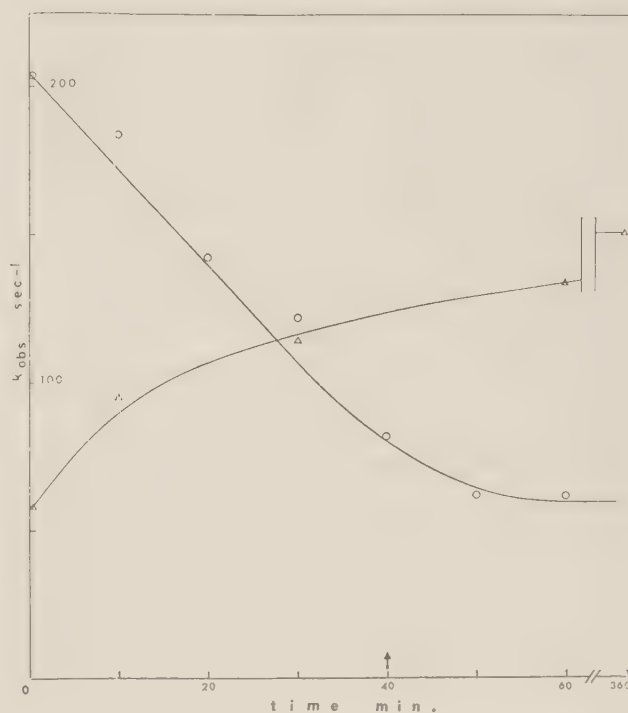


FIGURE 9: Inactivation (○) of N-guanidinated δ -chymotrypsin during reaction with citraconic anhydride, at pH 8, 0°. The arrow corresponds to the end of the addition of reagent. Reactivation (Δ) by treatment at pH 3, 25°. Activity measured toward *N*-Ac-L-TyrOEt. For experimental details, see text.

Unfortunately, $A_{16}ACT$, the final product obtained by acetylation of ACT was a mixture. It was found that the amount of residual ACT and of denatured protein was constant. This was confirmed by different methods. The study of the time dependence of acetylation of ACT showed that there was a good correlation between [^{14}C]acetyl incorporation, disappearance of free N-terminal isoleucine, and loss of enzymic activity toward *N*-Ac-L-TyrOEt. The observed properties of the final product $A_{16}ACT$ suggested that: (1) the activity toward specific substrates was due only to the 10% of residual ACT; (2) the 20% of denatured protein did not show any activity toward either *N*-Ac-L-TyrOEt or NPA; (3) the major component of the mixture, acetylated on Ile-16, was inactive toward specific substrates though active toward NPA and DFP. Since attempts to eliminate the contaminants were not successful, the mixture $A_{16}ACT$ was studied to determine the properties of the major component.

It has been demonstrated previously that a transition in chymotrypsin conformation was linked to the ionization of the α -amino group of Ile-16 (Oppenheimer *et al.*, 1966; Karibian *et al.*, 1968; Mc Conn *et al.*, 1969; Garel and Labouesse, 1970). This transition did not take place in $A_{16}ACT$, the specific optical rotation of which was constant with pH and corresponded to that of chymotrypsin at high pH. Circular dichroic spectra in the region of the dichroic band near 230 nm showed analogous features; the ellipticity of $A_{16}ACT$ at 230 nm did not depend on pH and was close to that of ACT at high pH.

It had been proposed that the "alkaline" conformation of chymotrypsin is equivalent to the conformation of chymotrypsinogen (Sigler *et al.*, 1968; Mc Conn *et al.*, 1969). Polarimetric and dichroic studies were not sensitive enough to distinguish between these two proteins and $A_{16}ACT$. However, the pK 's of both exposed tyrosines are different in ACTG and in the high pH form of ACT (Karibian *et al.*, 1968), showing a slight difference in conformation. The titration curve of tyrosines of $A_{16}ACT$ indicated that the protein acetylated on Ile-16 behaved more like ACT. The conformation of the protein acetylated on Ile-16 thus looked very similar to that of the alkaline form of chymotrypsin. This was supported by the recovery of the general kinetic and conformational properties of the native enzyme, upon the deacylation of the critical amino group.

The results of studies performed with nonspecific substrates corroborated the difference between ACTG and the protein acetylated on Ile-16 or ACT at high pH. ACTG did not appreciably hydrolyze NPA while the acetylated enzyme was reactive toward NPA and DFP, as chymotrypsin is reactive toward DFP up to pH 10 (Moon *et al.*, 1965).

Though the acetylation of the N-terminal α -amino group did not abolish the ability of the enzyme to hydrolyze NPA or react with DFP, this chemical modification had an effect on the rates of both reactions. For instance, the rate constants k_2 and k_3 involved in the reaction of chymotrypsin with NPA decreased by a factor of about 200 and about 3, respectively, upon acetylation of Ile-16.

The pH dependence of NPA hydrolysis by chymotrypsin was already studied, at least up to pH 9.5 (Bender *et al.*, 1966b); above pH 8, k_2 decreased while k_3 and K_m remained constant. The results obtained here for $A_{16}ACT$ suggested that this pH-dependent decrease of k_2 has a limit, which

should correspond to the acylation constant found for the enzyme with acetylated Ile-16.

At high pH the change in the ratio between k_2 and k_3 should result in an apparent decrease in the concentration of active sites when measured by the burst of *p*-nitrophenol. This was indeed observed for the protein acetylated on Ile-16.

It seems reasonable to assume that the ability of this enzyme to hydrolyze NPA and DFP reflects rather a preservation of the relative geometry of the catalytic residues Asp-102, His-57, and Ser-195 (Blow *et al.*, 1969) than the integrity of the whole native structure. The comparison of the acylation rate constants suggests that the conformation of the active site could be slightly distorted in the protein acetylated on Ile-16 or in the alkaline form of the enzyme. However, this distortion is not dramatic, since any important change in the geometry of the catalytic site should have an effect greater than a 200-fold decrease in acylation rates (Koshland and Neet, 1968). The suppression of the salt bridge between Ile-16 and Asp-194 (Matthews *et al.*, 1967) can be considered as noncritical in itself for the conformation of the catalytic site.

Although the active site of the enzyme acetylated on Ile-16 is capable of catalysis, this enzyme cannot hydrolyze either ester or amide substrates. A lack of binding or a nonproductive binding may then be the reason which prevents both steps of acylation and deacylation to take place. In fact, the affinity for specific substrates of the enzyme acetylated on Ile-16 should be comparable to that of the alkaline form of ACT, *i.e.*, about three orders of magnitude smaller than that of ACT at neutral pH (Johnson and Knowles, 1966; Garel and Labouesse, 1970). The specific substrates of chymotrypsin are not soluble enough to reach a concentration where an eventual activity of the alkaline form of ACT could be detected. It is possible that a limited area of the binding site is required for nonspecific substrates such as NPA and DFP, while the whole intact site is needed for specific bulky substrates (Knowles, 1965; Steitz *et al.*, 1969). The "alkaline transition" distorts a part of this binding site and leads to an enzyme which does not recognize anymore its specific substrates. The same type of discrimination between specific and nonspecific substrates has been already described for another chemically modified enzyme, thiol-subtilisin (Neet and Koshland, 1966; Polgar and Bender, 1967).

An hypothesis on the position of Ile-16 may also be drawn from this work. The tridimensional structure of chymotrypsin shows that in the neutral form the amino group of Ile-16 is buried inside the molecule (Matthews *et al.*, 1967; Sigler *et al.*, 1968), where it should be inaccessible to bulky acylating reagents like maleic or citraconic anhydrides. Therefore, to be acylated this amino group has to move out from its site and it remains outside, once acylated. In the high pH form of chymotrypsin, Ile-16 seems then to sit outside. This is also supported by the pK of its amino group, in ACT, 7.2 at 15° (J. R. Garel, 1969, unpublished results; Garel and Labouesse, 1970). The deprotonation of the N-terminal amino group of chymotrypsin corresponds thus to the initiation of a conformational rearrangement of the protein. This transition involves a movement of the polypeptide chain bearing Ile-16 from the inside of the hydrophobic cleft toward the outside of the molecule, and the

distortion of the binding site or the partial masking of the catalytic site.

The present work shows that chymotrypsinogen, the "neutral" form and the "alkaline" form of chymotrypsin have different conformations. In the zymogen the bond between Arg-15 and Ile-16 has to be accessible to trypsin (Rovero and Desnuelle, 1953; Dreyer and Neurath, 1955) and thus Ile-16 sits necessarily outside the molecule. One may assume that the splitting of this bond leads to a protein which is chymotrypsin in the alkaline form. This first irreversible step could result in either the creation of the catalytic site or its partial unmasking. The hypothesis of a partial unmasking is supported by the fact that only small structural differences are found between chymotrypsinogen and the alkaline form of chymotrypsin. The active site would thus preexist in the zymogen.

The protonation of Ile-16 could control the second reversible step of activation which would result in the proper adjustment of the binding site. The conformational changes linked to this activation process (Neurath *et al.*, 1956; Fasman *et al.*, 1966) could correspond to an outside to inside movement of Ile-16, in order to make possible the electrostatic interaction between its α -amino group and the β -carboxyl of Asp-94. The resulting salt bridge may then be used by the protein to lock the active conformation.

Acknowledgments

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Added in Proof

Since the submission of this paper, Freer *et al.* (1970) have reported the 2.5-Å crystal structure of chymotrypsinogen. Their findings about the preformation of the catalytic site in the zymogen and the different position of Ile-16 in chymotrypsinogen and in the active enzyme support the hypotheses presented here.

References

- Bender, M. L., Begue-Canton, M. L., Blakeley, R. L., Brubacher, M. J., Feder, J., Günter, C. R., Kézdy, F. J., Killheffer, J. V., Jr., Marshall, T. H., Miller, C. G., Roseske, R. W., and Stoops, J. K. (1966a), *J. Amer. Chem. Soc.* 88, 5890.
- Bender, M. L., Gibian, M. J., and Whelan, D. J. (1966b), *Proc. Nat. Acad. Sci. U. S.* 56, 833.
- Bender, M. L., and Nakamura, K. (1962), *J. Amer. Chem. Soc.* 84, 2577.
- Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London)* 221, 337.
- Bray, G. A. (1960), *Anal. Biochem.* 1, 279.
- Bruice, T. C., and Benkovic, S. J. (1966), *Bioorganic Mechanisms*, Vol. I, New York, N. Y., W. A. Benjamin Inc., p 62.
- Butler, P. J. G., Hartley, B. S., Harris, J. I., and Lieberman, R. (1969), *Biochem. J.* 112, 679.
- Chervenka, C. H., and Wilcox, P. E. (1956), *J. Biol. Chem.* 222, 635.
- Dixon, H. B. F., and Perham, R. N. (1968), *Biochem. J.* 109, 312.
- Dreyer, W. J., and Neurath, H. (1955), *J. Biol. Chem.* 217, 527.
- Edsall, J. T., and Wyman, J. F. (1958), *Biophysical Chemistry*, Vol. I, New York, N. Y., Academic, p 450.
- Erlanger, B. F., and Edel, F. (1964), *Biochemistry* 3, 346.
- Faller, L., and Sturtevant, J. M. (1966), *J. Biol. Chem.* 241, 4825.
- Fasman, G. D., Foster, R. J., and Beychok, S. (1966), *J. Mol. Biol.* 19, 240.
- Freer, S. T., Kraut, J., Robertus, D. J., Wright, H. T., and Xuong, H. Ng. (1970), *Biochemistry* 9, 1997.
- Garel, J. R., and Labouesse, B. (1970), *J. Mol. Biol.* 47, 41.
- Ghélis, C., Labouesse, J., and Labouesse, B. (1967), *Biochem. Biophys. Res. Commun.* 29, 101.
- Gutfreund, H., and Sturtevant, H. M. (1956), *Biochem. J.* 63, 656.
- Haugland, R. P., and Stryer, L. (1967), *Conform. Biopolym. Pap. Int. Symp.*, 321.
- Hess, G. P., Mc Conn, J., Ku, E., and Mc Conkey, G. (1970), *Phil. Trans. Roy. Soc. London* 257, 89.
- Himoe, A., Parks, P. C., and Hess, G. P. (1967), *J. Biol. Chem.* 242, 919.
- Jencks, W. P., and Gilchrist, M. (1964), *J. Amer. Chem. Soc.* 86, 5616.
- Johnson, C. H., and Knowles, J. R. (1966), *Biochem. J.* 103, 428.
- Karibian, D., Laurent, C., Labouesse, J., and Labouesse, B. (1968), *Eur. J. Biochem.* 5, 260.
- Katz, J., Lieberman, I., and Barker, H. A. (1953), *J. Biol. Chem.* 200, 417.
- Knowles, J. R. (1965), *J. Theor. Biol.* 9, 213.
- Koshland, D. E., Jr., and Neet, K. E. (1968), *Annu. Rev. Biochem.* 37, 359.
- Labouesse, J., and Gervais, M. (1967), *Eur. J. Biochem.* 2, 215.
- Lawson, W. B., and Schramm, H. J. (1965), *Biochemistry* 4, 377.
- Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature (London)* 214, 652.
- Mc Conn, J., Fasman, G. D., and Hess, G. P. (1969), *J. Mol. Biol.* 39, 551.
- Moon, A. Y., Sturtevant, J. M., and Hess, H. P. (1965), *J. Biol. Chem.* 240, 4204.
- Moore, S., and Stein, W. N. (1948), *J. Biol. Chem.* 176, 367.
- Neet, K. E., and Koshland, D. E., Jr. (1966), *Proc. Nat. Acad. Sci. U. S.* 56, 1606.
- Neurath, H., Rupley, J. A., and Dreyer, W. J. (1956), *Arch. Biochem. Biophys.* 65, 243.
- Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966), *J. Biol. Chem.* 241, 270.
- Philipps, D. M. P. (1958), *Biochem. J.* 68, 35.
- Polgar, L., and Bender, M. L. (1967), *Biochemistry* 6, 610.
- Rovero, M., and Desnuelle, P. (1953), *Biochim. Biophys. Acta* 13, 300.
- Schoellmann, G., and Shaw, E. (1962), *Biochem. Biophys. Res. Commun.* 7, 36.
- Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta* 16, 570.

Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol.* 35, 143.
 Steitz, T. A., Henderson, R., and Blow, D. M. (1969), *J. Mol. Biol.* 46, 337.

Tanford, C. (1961), *J. Amer. Chem. Soc.* 83, 1628.
 Valenzuela, P., and Bender, M. L. (1969), *Proc. Nat. Acad. Sci. U. S.* 63, 1214.
 Volini, M., and Tobias, P. (1969), *J. Biol. Chem.* 244, 5105.

